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TITLE: Regulation of Breast Cancer Cell Motility by Golgi-Mediated Signaling

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  We have previously determined that the Rho-specific guanine nucleotide exchange factor, Dbs, regulates both normal and tumor cell motility. Since full-length Dbs localizes to the Golgi, we have determined whether Golgi-mediated signaling activities are required for breast cancer cell movement. Thus, stable MDA-MB-231 cell lines were established in which the activity of Golgi-localized Dbs was suppressed by a novel Sec14 domain-mediated genetic inhibitor. The level of endogenous activated Cdc42 was reduced in these cells suggesting that one function of Dbs is to regulate Golgi-localized Cdc42. Although the cells exhibited their normal mesenchymal morphology, they were impaired in their motility as measured by wound healing and transwell assays. Interestingly, the impairment was in directional migration, rather than cell movement per se. The cells were unable to reorient their Golgi in response to serum stimulation which likely accounts for the defect in directional movement.					
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## FINAL REPORT

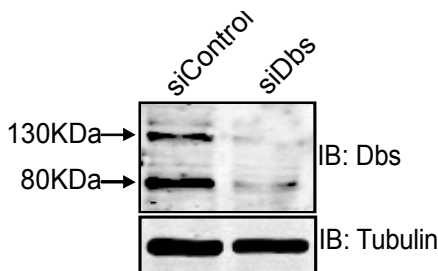
### Introduction:

The Rho family GTPase, CDC42, is found at the leading edge of motile cells where it regulates both lamellipodia formation and microtubule capture, and in the Golgi where it regulates organelle reorientation and secretion. The multiple functions of CDC42 are regulated in part by guanine nucleotide exchange factors (GEFs), which convert it from an inactive to an active conformation. Dbs is a CDC42-specific GEF that is anchored in the Golgi by its amino-terminal, lipid binding, Sec14 domain. Although the function of Dbs in the Golgi is unknown, it has been shown to target CDC42, and support motility in both Schwann cells and breast tumor cell lines (1). This suggests that Golgi membranes are platforms for activities that support motility, and that these activities could be targeted in breast cancer. Additionally, since RhoGEFs are more restricted in their cellular distribution than their RhoGTPase substrates, they may provide opportunities to specifically target tumor cell motility without disrupting more fundamental aspects of cell behavior. The goals of this proposal were relatively straightforward; to generate cell lines in which the expression, or activity, of Golgi-localized Dbs was stably suppressed, and then examine these cell lines in cell- and animal-based assays for motility and invasion. Suppression of Dbs activity would be accomplished through targeting shRNAs, or by use of a Sec14 domain-based genetic inhibitor that we have previously described.

### Body:

#### **Task 1a and 1b: Construct shRNA vectors for stable suppression of Dbs and Cdc42, and select cell lines for use in transwell and live animal imaging assays.**

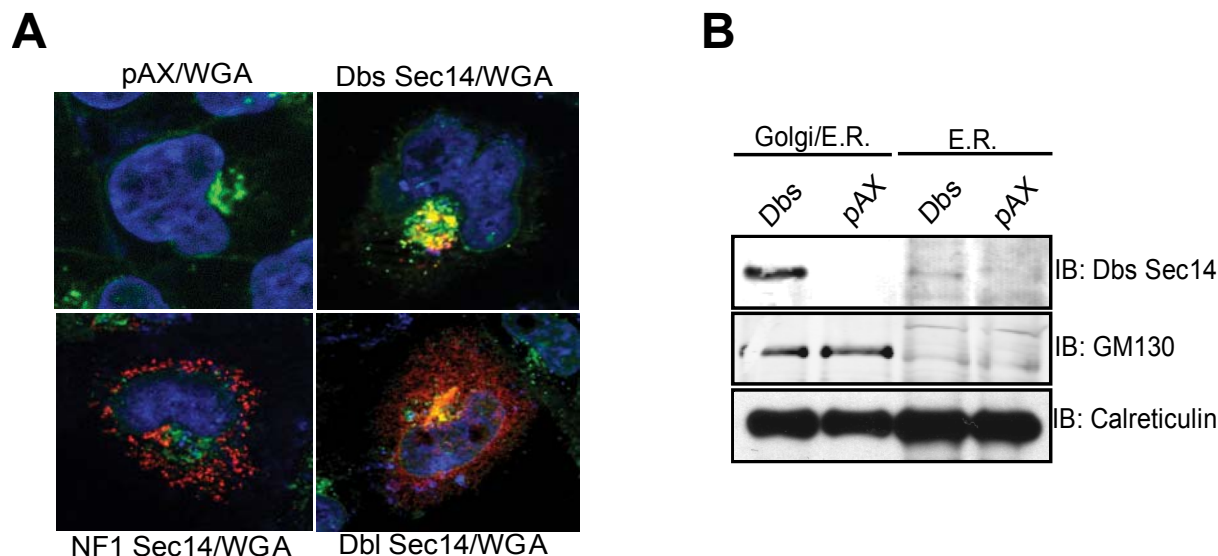
In order to stably suppress Dbs expression in the highly motile MDA-MB-231- D3H1 breast tumor cell line, shRNAs that specifically target Dbs were designed and synthesized, and then cloned into the pSUPER mammalian expression vector. We had previously observed that Dbs is expressed as an 80kD isoform in the cytoplasm, and a 130kD isoform in the Golgi (see Figure 1). Since the goal was to specifically suppress the expression of the 130kD form, eight shRNA target sequences were identified along the full-length of the mRNA. Cell lines were established that express the shRNAs, and then examined by Western blot for stable suppression of Dbs. Although we were able to establish a cell line in which Dbs expression was almost completely suppressed (Figure 1), we were unable to specifically suppress expression of the 130kD band. To circumvent this problem a second set of shRNAs was ordered and tested, but with identical results. Since this cell line would not allow us to distinguish between effects due to cytoplasmic Dbs, and those due to Golgi-localized Dbs, we decided to drop this approach, and focus our efforts on the Golgi-specific Sec14 domain-based inhibitor.



**Figure 1: shRNAs that target Dbs reduce the expression of both the 80kD and 130kD isoforms in MDA-MB-231 cells.** Cell lines were selected that express control or Dbs-specific shRNAs. Lysates were collected and examined by Western blot for expression of endogenous Dbs. Tubulin was used as a loading control.

When transiently overexpressed, the isolated Sec14 domain of Dbs accumulates in the perinuclear region where it is thought to bind to endogenous full-length Dbs and block its transforming activity (2).

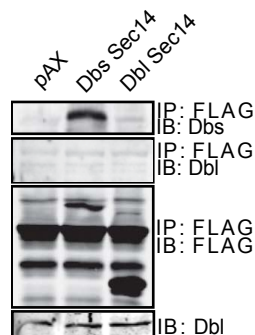
As an alternative to shRNAs, MDA-MB-231 cell lines were established that stably express this inhibitor. To confirm that the inhibitor associates specifically with the Golgi compartment, co-immunofluorescence was performed with a Golgi-specific marker (Figure 2A), and organelle fractionation experiments were conducted (Figure 2B). These studies confirmed the presence of the inhibitor in the Golgi, and its exclusion from the endoplasmic reticulum and the cytoplasm. These cell lines were selected for further analysis.



**Figure 2: The isolated Sec14 domain of Dbs localizes to the Golgi.** (A) Cells were stably transfected with the indicated FLAG-tagged constructs and then examined by immunofluorescence for cellular distribution of the overexpressed proteins. Co-localization of the indicated Sec14 domains with the Golgi was demonstrated using the WGA marker. (B) Sub cellular fractionation of cells stably transfected with the Dbs Sec14 domain or cognate vector (pAX). Fractions were western blotted for the over expressed protein, and both Golgi and ER markers (GM130 and calreticulin).

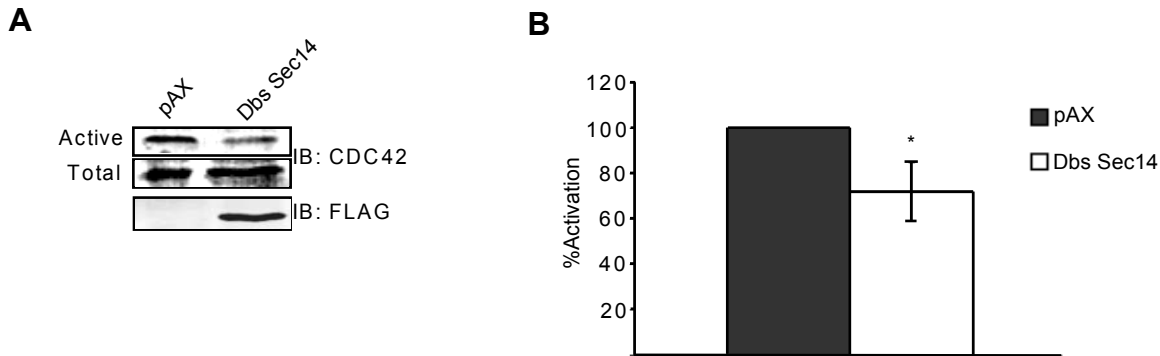
### Task 1c: Validate cell lines using western blots and affinity precipitation assays.

Next we wished to confirm that the Dbs inhibitor was targeting the function of endogenous Dbs in the Golgi. First we determined whether the inhibitor directly interacted with endogenous Dbs. Thus, stable cell lines that express the isolated Sec14 domain of Dbs, or the related RhoGEF Dbl, were used for co-immunoprecipitation (Figure 3). Immunoprecipitates were collected and examined for expression of endogenous Dbs or Dbl. This analysis showed that the Sec14 domain inhibitor binds directly to endogenous Dbs, but not to endogenous Dbl.



**Figure 3: The isolated Sec14 domain interacts with endogenous Dbs, but not Dbl.** Cells were stably transfected with the isolated FLAG-tagged Sec14 domain of Dbs, Dbl, or cognate vector (pAX) as indicated. Lysates were collected at 48 hr and examined by western blot for expression of endogenous Dbl (lower blot). IP indicates antibody used for immunoprecipitation while IB indicates an antibody used for a Western blot.

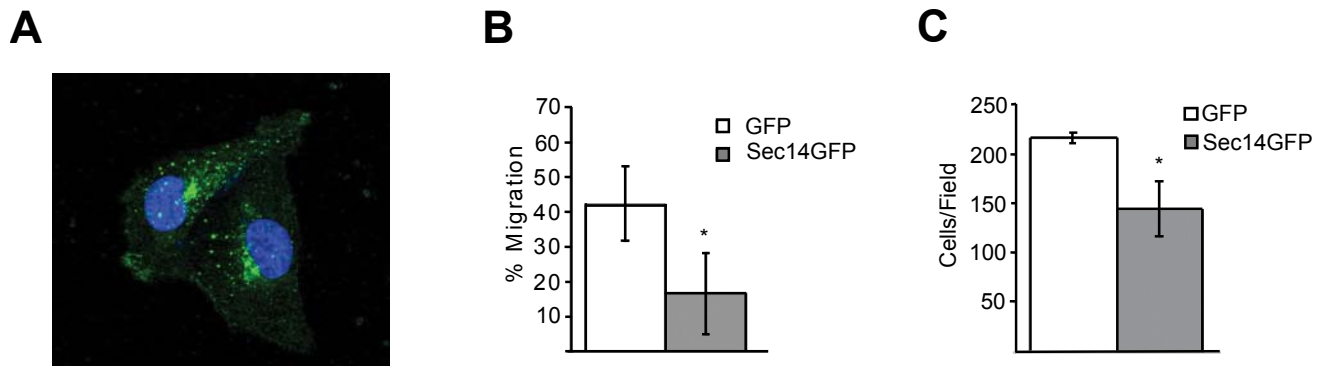
Next we determined whether the interaction between the Sec14 domain inhibitor, and endogenous Dbs, can influence the levels of activated endogenous Cdc42. For this analysis, cells that stably express the isolated Sec14 domain were subject to affinity precipitation assays. This analysis revealed that the level of active Cdc42 was reduced by approximately 25% in the presence of the inhibitor. This suggests that Dbs is regulating the endogenous pool of Cdc42 that is localized to the Golgi compartment, and we can target this activity using the Sec14 domain inhibitor.



**Figure 3: The isolated Sec14 domain inhibits endogenous Cdc42 activity.** Lysates were collected and examined by western blot for expression of endogenous Cdc42 (total) and overexpressed Sec14 (FLAG). Lysates were then subjected to affinity purification with GST-PAK3. (A) GTP-bound Cdc42 (active) was visualized by Western blot. (B) Quantification of band intensity was done using the Odyssey infra-red imager (Li-Cor). Results are expressed as the ratio of intensity of activated Cdc42 to total Cdc42 averaged from 3 independent experiments  $\pm$  S.D.

#### Task 2a: Compare cell lines in transwell motility and invasion assays

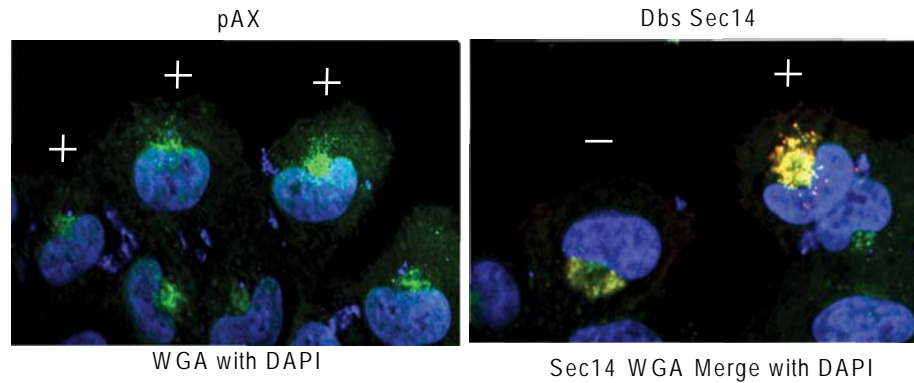
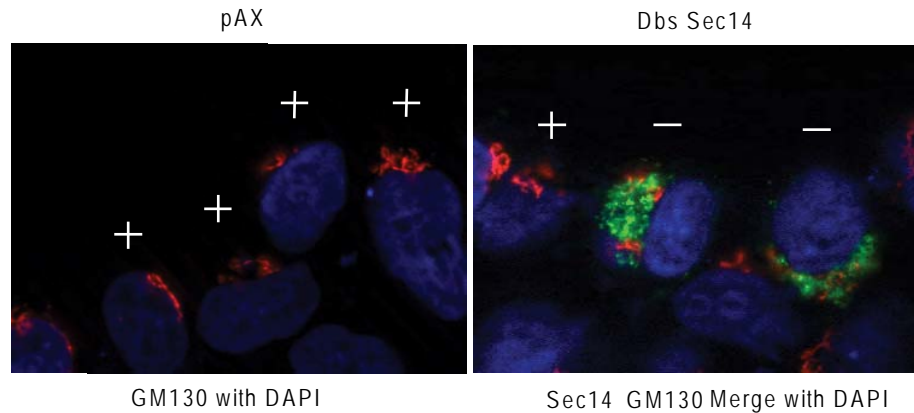
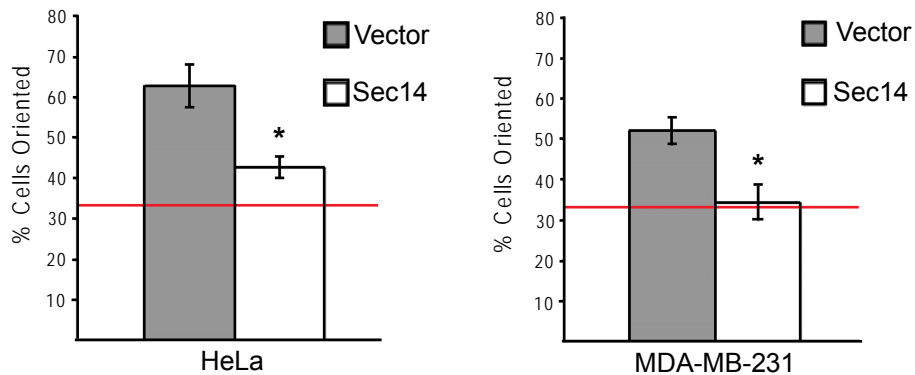
Our analysis of the stably transfected MDA-MB-231 cell lines revealed that the Sec14 domain inhibitor was localized to the Golgi apparatus (Figure 4A) where it interfered with Dbs function, and limited Cdc42 activation. Next we determined whether this was sufficient to influence the motile phenotype of these cells. For this analysis two assays were utilized, the wound healing assay (Figure 4B) and the transwell motility assay (Figure 4C). In order to visualize live cells that express the inhibitor, the isolated Sec14 domain was fused to GFP. Cells expressing the GFP protein alone were readily able to migrate into an open wound after 19 hours. However, when cells expressed the Dbs Sec14-GFP fusion protein, motility was significantly impaired (Figure 4B). The effect of the inhibitor on motility was also observed in a transwell motility assay (Figure 4C). Cells were stably transfected with the indicated constructs and then FACS sorted based on GFP expression. Using this approach we observed that cells that express the inhibitor show an approximately 35% reduction in motility relative to cells that express GFP alone. Since this is similar to what we previously observed using siRNAs that target Dbs (1), we conclude that the Golgi-localized Dbs protein is the primary pool of endogenous Dbs that controls cell motility. Interestingly, we did not observe any difference in morphology between cells that express the inhibitor and those that express GFP alone.



**Figure 4: Golgi-localized Dbs supports breast cancer cell motility.** (A) Distribution of the Dbs inhibitor in MDA-MB-231 cells. Cells were examined by immunofluorescence to determine the cellular distribution of the inhibitor. (B) MDA-MB-231 cells were stably transfected with the GFP-Sec14 domain of Dbs, or GFP alone, and then examined in a wound healing assay. The number of GFP positive cells that migrated into the wound was calculated at 19 hr and expressed as a percentage of total GFP positive cells at the wound edge. (C) MDA-MB-231 cells were stably transfected with the GFP-Sec14 domain of Dbs, or GFP alone, and then sorted by GFP and examined in a transwell motility assay on filters pre-coated with type I collagen. The motility of cells expressing Dbs Sec14 domain is expressed relative to that of cells expressing cognate vector. Nine fields of view were scored from 3 independent transwells for both transfected groups of cells. Results are expressed as the average number of cells counted in each field of view  $\pm$  S.D. of three independent experiments performed in triplicate.

#### **Task 2b: Evaluate cell lines for invasion velocity and movement type using confocal microscopy**

Despite the significant reduction in number of cells that traversed the filter in transwell assays, many cells were clearly still able to move in the presence of the inhibitor. In addition, the morphology of the cells did not change – they remained mesenchymal in appearance. This was confirmed by our inability to detect any difference in invasion velocity. Thus, we explored the possibility that Dbs may be required for directed migration, but not random movement. Since directed motility in mammalian cells typically requires repositioning of the Golgi towards the leading edge (3) we determined whether impaired motility was associated with a failure to orient the Golgi towards the wound (Figure 5). For this analysis, HeLa and MDA-MB-231 cells were stably transfected with the inhibitor, or cognate vector. Cultures were wounded and then allowed to reorient for 3 hours. Cells were then fixed, and stained with a marker for Golgi and the overexpressed Sec14 domain. Both the WGA Golgi marker (Figure 5A) and GM130 Golgi marker (Figure 5B) were used to monitor orientation. In control cells approximately one third of the cells are orientated towards the Golgi prior to serum stimulation, which represents the expected random probability (the horizontal lines in Figure 5C represent this baseline). Three hours after wounding the percentage of control cells in which the Golgi is orientated towards the wound increases to approximately 60% in both cell types. This reorientation is substantially reduced if the Sec14 inhibitor suppresses Dbs expression. This suggests that Dbs-mediated activation of Cdc42 in the Golgi apparatus is required to support directed migration, but not overall cell movement, per se. Since Golgi reorientation is thought to be regulated by events at the leading edge, this represents a novel function for Dbs and Cdc42 in the Golgi.

**A****B****C**

**Figure 5: Dbs supports Golgi-reorientation during directed migration** HeLa and MDA-MB-231 cells were examined for serum-dependent Golgi reorientation in a wound healing assay. Confluent cells were transfected with the Dbs inhibitor or cognate vector overnight and then wounded. After wounding, cells were cultured in 10% serum for 3 hr and then fixed and stained for Sec14 domain, Golgi marker (WGA for A and GM130 for B) and DAPI (blue). A and B, greater than 50 cells at the wound edge were then scored as Golgi orientated (+) or not orientated (-). Representative HeLa cells are shown. (D) data are represented as the average fraction of oriented cells from three independent experiments  $\pm$  S.D. Horizontal lines indicate the fraction predicted to be orientated by random chance.



### **Task 3: Evaluate cell lines in the orthotopic mouse model**

Because of the unanticipated difficulty in generating cell lines in which the endogenous function of Dbs could be stably suppressed by shRNAs, it took longer than expected to generate cell lines that could be examined in the orthotopic mouse model. For reasons described above, it was necessary to abandon the shRNA-based strategy, and focus our attention on the Golgi-specific Sec14 domain-based inhibitor that we had described in our grant application. Stable cell lines that express this inhibitor were generated. Although this cell line has now been fully characterized, and is suitable for this analysis (see above), this study has not yet been completed. We anticipate that these studies will be completed over the next 1-3 months.

### **Key Research Accomplishments**

- We have identified a genetic inhibitor that specifically interacts with, and targets, endogenous, Golgi-localized Dbs.
- We have established stable cell lines in which the endogenous function of Golgi-localized Dbs is suppressed.
- We have determined that Dbs regulates a Golgi-localized, endogenous pool of Cdc42.
- We have determined that overexpressed, Golgi-localized Dbs and Cdc42 support breast tumor cell motility
- We have determined that Golgi-localized Dbs does not regulate cell movement type or velocity.
- We have determined that Golgi-localized Dbs and Cdc42 regulates Golgi-reorientation during directed migration in breast tumor cells

### **Reportable Outcomes**

#### Meeting Abstracts and Publications

Fitzpatrick, E., E. Kostenko, and I.P. Whitehead (2010) The role of the guanine nucleotide exchange factor Dbs in Golgi-ER dynamics. Annual Retreat of the New Jersey Commission on Cancer Research, New Brunswick, NJ.

#### Personnel Receiving Pay from Research effort

Ian P. Whitehead, Principal Investigator  
Ru Chen, Research Associate

### **Conclusion**

During this study we established cell lines in which the activity of endogenous Dbs is stably suppressed. We have utilized these cell lines to identify the role of Dbs-mediated Golgi-dynamics in breast tumor cell motility. Although the cell lines appear morphologically normal relative to vector controls, they exhibit impaired motility in both wound healing and transwell motility assays. This is associated with decreased activity of the small GTPase, Cdc42. Although a small pool of Cdc42 has previously been identified in the Golgi, the identity of its GEF had not been previously determined, nor had its role in cell motility. The most intriguing observation is the demonstrated role for Dbs in Golgi-mediate reorientation during cell migration. The possibility that such signals emanate from the Golgi itself, and are not mediated in an indirect manner by activity at the leading edge, has not been previously explored. This link suggests that inhibitors that specifically target Golgi-mediated activity can be used to control the motile phenotype of breast tumor cells.

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